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Quantitative DNA fiber mapping

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The assembly of sequence ready, high-resolution physical maps and construction of minimally overlapping contigs for the human as well as model genomes requires accurate determination of the extent of overlap between adjacent clones as well as their relative orientation. This is presently done by procedures such as clone fingerprinting, Southern blot analysis or clone end sequencing. We present a complementary analytical technique to map directly cloned DNA sequences on to individual stretched DNA molecules. This approach uses the hydrodynamic force of a receding meniscus to prepare straight high molecular weight DNA molecules that provide a linear template of ~2.3 kb/μm on to which the cloned probes can be mapped by *in situ* hybridization. This technique has numerous advantages such as a very high density of mapping templates, reproducible stretching of the mapping template providing a linear genomic scale, determination of clone orientation and direct visualization of DNA repeats. The utility and accuracy of quantitative DNA fiber mapping are illustrated through three examples: (i) mapping of lambda DNA restriction fragments along linearized ~49 kb long lambda phage DNA molecules with ~1 kb precision; (ii) localization of the overlap between a cosmid and a colinear P1 clone; and (iii) mapping of P1 clones along a ~490 kb yeast artificial chromosome (YAC) with ~5 kb precision and estimation of the ~25 kb gap between them.

INTRODUCTION

Efficient construction of high-resolution physical maps of the human genome and genomes of model organisms in a form suitable for DNA sequencing is essential for the human genome project (1). Such maps are typically comprised of overlapping DNA fragments cloned into cosmids, P1s, bacterial artificial chromosomes (BACs) or other vectors that are amenable to sequencing (2–5). Assembly of these maps requires identification of overlapping DNA clones, assessment of the degree of overlap between clones and identification and characterization of the extent of gaps between adjacent but non-overlapping contigs. This has been accomplished by various forms of clone fingerprinting, by hybridization to cloned sequences arrayed on filters and by identification of clones carrying common sequence tagged sites (STSs) (5–10). These techniques are limited, however, because they do not readily yield information about contig orientation, extent of overlap of contig elements or provide information about the extent of gaps between contigs.

Fluorescence *in situ* hybridization (FISH) provides additional information for physical map assembly. For example, FISH to metaphase chromosomes allows localization and ordering of cloned DNA fragments with few-megabase resolution (11) and FISH to interphase nuclei allows probes to be ordered with ~50 kb resolution (12,13). However, these techniques do not provide precise information about the extent of clone overlap or about the separation between elements in the map with high resolution. This limitation has been partly removed using FISH with clones to be ordered to preparations of DNA fibers from decondensed nuclei (14–19) or isolated cloned DNA (20). These techniques allow visualization of probe overlap and provide semi-quantitative information about the existence and size of gaps in the map. However, the DNA in fiber preparations used to date have not been optimal for quantitative analysis because the fibers on to which clones are mapped are condensed to varying degrees and because useful DNA fibers have been hard to find.

The recent work of Bensimon *et al.* (21) demonstrated that the non-uniform condensation of DNA fiber preparations can be minimized by employing molecular combing. In this process, a solution of target DNA molecules on to which probes are to be mapped, is placed on a flat glass surface prepared so that the DNA molecules randomly attach at one or occasionally at both ends. The solution is then covered with a coverslip and allowed to dry. The DNA molecules are straightened and uniformly stretched at ambient temperature during drying by the hydrodynamic action of the receding meniscus. Bensimon *et al.* (21) showed that molecules prepared in this manner are remarkably straight and homogeneously stretched (~2.3 kb/μm). They also showed that molecules as long as 1 Mb can be straightened using molecular combing.

Here, we show that FISH and quantitative image analysis can be used to map clones with few kilobase resolution on to individual DNA molecules straightened by molecular combing, greatly facilitating physical map assembly. Reproducible DNA stretching and the high efficiency of DNA hybridization allows accurate mapping using only a few fibers. These are easily found because combing results in a high density of linearized DNA fibers. Thus, only a few microscopic fields of view need to be examined.

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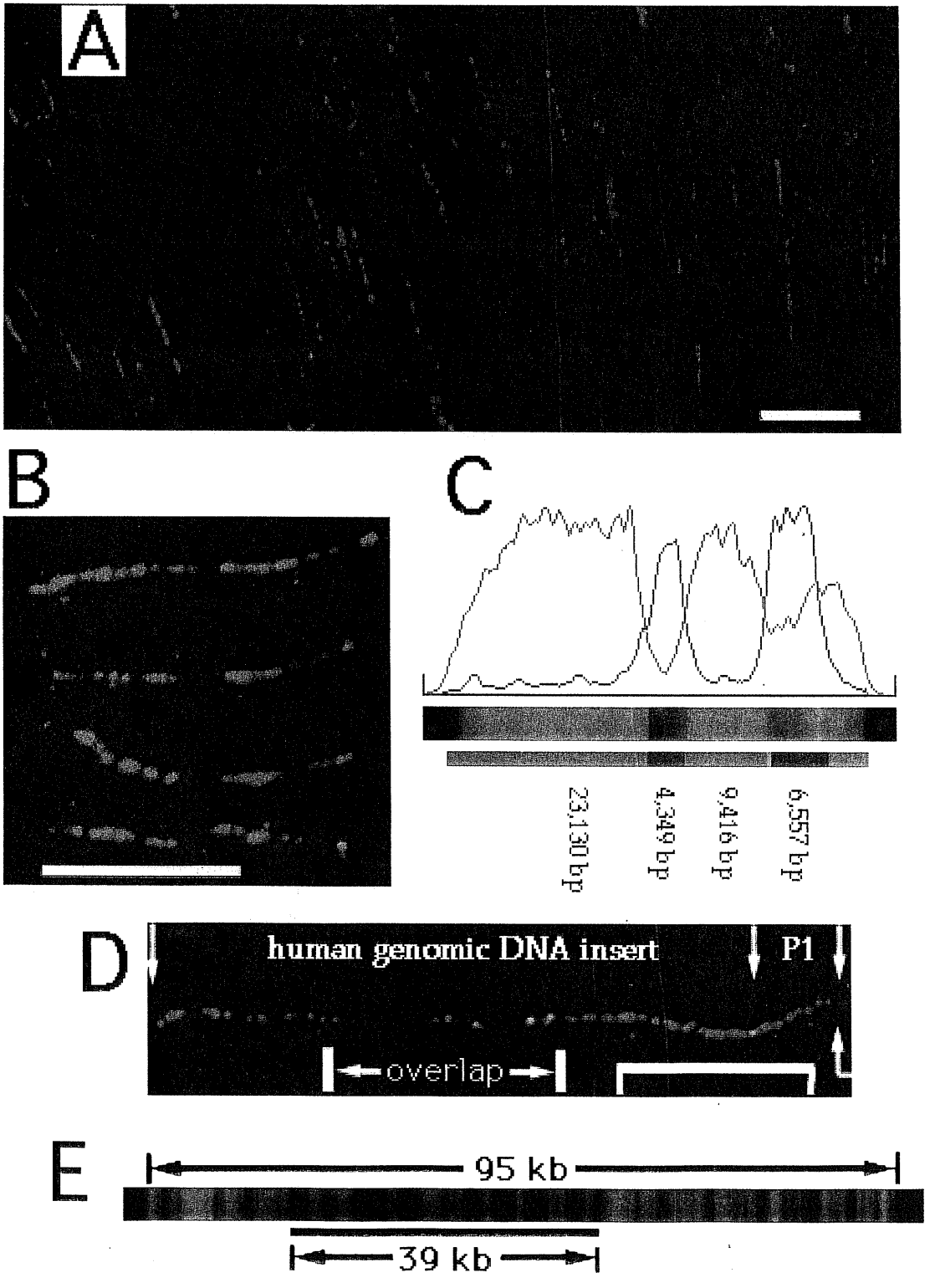
RESULTS

Molecular combing was accomplished using a variation of the procedure described by Bensimon *et al.* (21). Treatment of the glass substrate to which the DNA fibers are attached so that they bind preferentially at their respective 3′- or 5′-end (or sometimes both) prior to stretching is a critical aspect of the procedure. Bensimon *et al.* (21) treated glass surfaces by silanation to expose vinyl groups, and proposed binding of the DNA via 5′-phosphates (21). We examined several different surfaces including vinyl-, epoxy- or amino-derivatized glass substrates (22,23) and found that derivatization of standard



microscope slides or borosilicate coverslips with 3-aminopropyltriethoxy silane (APS) (23) resulted in production of linearly stretched DNA molecules during combing. The density of such molecules could be adjusted by altering the concentration of the DNA molecules prior to combing.

DNA molecules were suspended in a solution containing glycerol and *p*-phenylenediamine (AF-solution) to reduce breakage during combing, to minimize intermolecular binding to the solid substrate and to reduce photobleaching during fluorescence microscopy (24). This proved to be superior to suspension in water or other commonly used buffers (MES, MOPS, phosphate or borate buffer) as use of these resulted in excessive DNA fragmentation during combing. The binding of DNA and molecular combing were examined visually by staining the DNA with YOYO prior to the molecular combing process (21).

We assessed the accuracy and precision with which DNA could be mapped and sized using quantitative DNA fiber mapping by hybridizing 4.3, 6.6, 9.4 and 23 kb lambda DNA/*Hin*dIII fragments to combed lambda phage DNA. We used a commercially available preparation of high molecular weight lambda DNA of unknown integrity and deposited the equivalent of approximately 10^6 full length DNA molecules on APS-derivatized slides. The 9.4 and 23 kb probes were labeled with biotin and the 4.3 and 6.6 kb probes were labeled with digoxigenin and hybridized overnight to the combed fibers. The hybridized probes were then detected with avidin-FITC and rhodamine-labeled antidigoxigenin. Hybridization of these probes to combed phage DNA molecules is shown in Figure 1A. The hybridization efficiency was uniformly high and most molecules showed alternating red and green fluorescing domains in the regions homologous to the probes as expected (Figure 1B). The locations of the probes are precisely known from the phage DNA sequence and are illustrated in Figure 1C. Only 3–4% of the molecules were intact and showed the predicted hybridization pattern. The other molecules were shorter and showed hybridization pattern as expected from truncated lambda DNA molecules. This was not a limitation because of the high density of fibers. On average, we found several full length lambda molecules per field of view using a 63×microscope objective. The intact fibers were ~21.6 μm in length indicating that the 48.9 kb molecules were stretched to 2.26 kb/μm. This is very similar to the stretching described by Bensimon *et al.* (21).

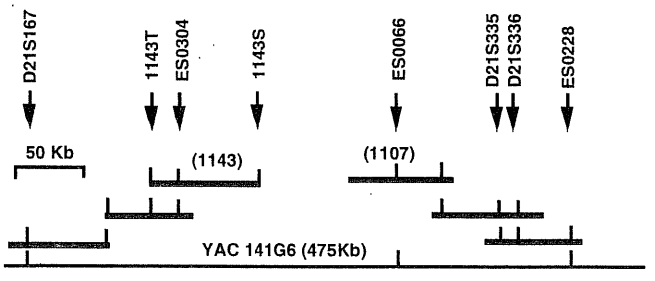


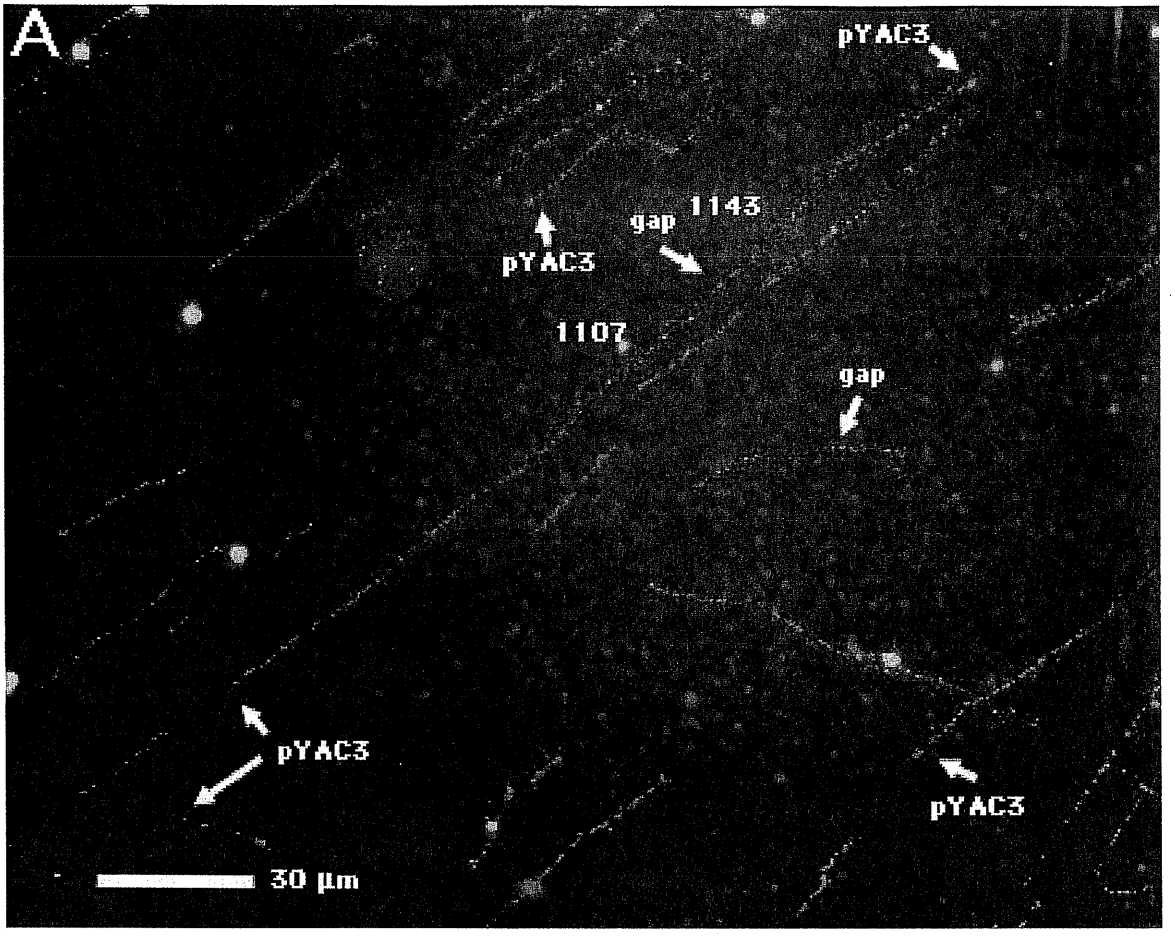
Figure 2. The map based on STS content mapping of P1 clones '1107' and '1143' and the YAC clone '141G6' shows their relative positions and the gap region between two flanking P1 contigs.

We quantitatively analyzed the reproducibility with which the probes could be mapped and sized by analyzing 45 different fibers of approximately full length using digital imaging microscopy. The mean distances of the boundaries of the 4.3, 6.6 and 9.4 kb *Hin*dIII fragments from the phage molecule endpoints were within ~2 kb of the known distances and the mapping precision was better than 3 kb for all measurements. The reproducibility of quantitative fiber mapping is illustrated in Figure 1C, which shows red and green profiles produced by averaging red and green fluorescence profiles measured from 45 individual DNA fibers.

The sizes of the internal 4.3, 6.6 and 9.4 kb fragments were estimated from measurements of the length of their hybridization domains along the 45 individual lambda molecules to be 4.1 ± 1.3 kb, 5.9 ± 1.5 kb and 9.7 ± 1.5 kb, respectively, assuming that the DNA molecules were reproducibly stretched to 2.26 kb/μm.

The increasing availability of mapped large insert clones (e.g. YACs, BACs and P1s) allows ready assembly of smaller insert sequence-ready contigs from the larger insert clones. In a typical study, the large insert clone either may be subcloned or used to select smaller insert clones from a genomic library. Either way, assembly of the resulting subclones into a minimally spanning contig is required for further analysis. Complete closure of the map requires identification and characterization of any gaps. We demonstrate the utility of FISH of the smaller clones to the larger combed DNA molecules in two examples: hybridization of a cosmid clone to a linearized P1 molecule and hybridization of P1 probes to a linearized YAC molecule.

Figure 1. Sequence-specific labeling of straightened DNA molecules by hybridization. (**A**) Lambda DNA was deposited on amino-silanated glass slides and straightened by hydrodynamic action of a receding meniscus. Biotin- and digoxigenin-labeled probes were hybridized overnight and detected with avidin-FITC (green) and rhodamine-labeled antidigoxigenin (red), respectively. The hybridization of differently labeled *Hin*dIII fragments produced regions of alternating red and green fluorescence along lambda molecules. (**B**) Alignment of individual lambda DNA molecules demonstrates the reproducibility of the technique. The molecules are approximately full length and show the expected hybridization pattern. The green fluorescence distal to the 6557 bp fragment is due to impurities (partial digests) contained in the preparation of the 23 130 bp probe. (**C**) Longitudinal profiles of red and green fluorescence produced by averaging 45 lambda DNA molecules (top). Individual profiles were aligned to the center of the green fluorescing 9.4 kb fragment. Individual profiles were not normalized. Fluorescence profiles (top) were used to generate a color code bar (center). The bottom part shows the predicted hybridization domains based on the locations of *Hin*dIII restriction fragments in the phage DNA sequence. (**D**) Hybridization of cosmid DNA to stretched P1 DNA molecules delineates homologous regions. A probe generated from a cosmid clone is visualized in red. P1 specific probe (green) was hybridized to counterstain the P1 molecule. This image of a single hybridized P1 molecule is part of a larger field containing several P1 molecules. The region of overlap (red fluorescence) is indicated. A 493 bp region of high homology (h) is located close the unique *Not*1 site in p*Sac*BII P1. (**E**) Alignment of 11 individual P1 molecules hybridized with the cosmid probe (red) in combination with the P1 DNA (green). The homologous region is approximately 39 ± 4 kb based on the total length of the P1 molecule of 95 kb. The sizemarker bars represent 10 μm.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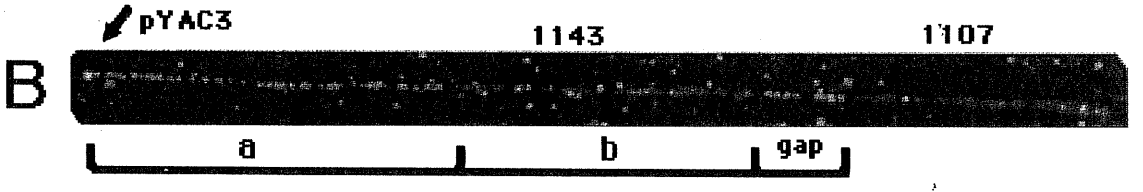
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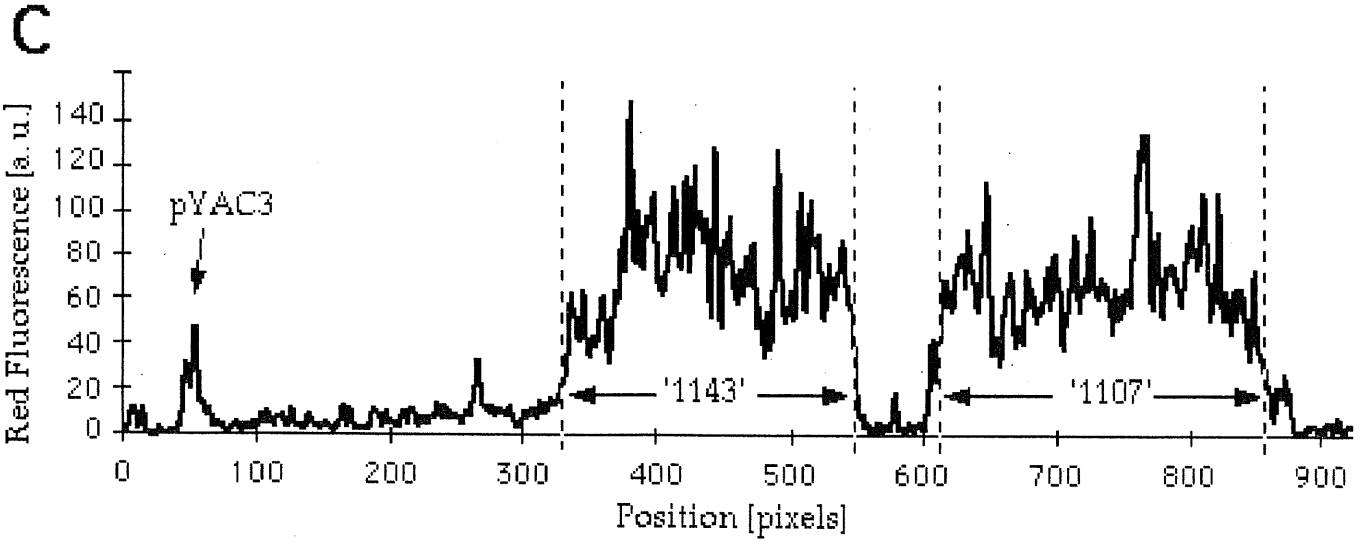
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Clone RMC17P036 from the DuPont P1 library (4) contains an ~80 kb segment of the human p53 gene. We mapped a 39 kb cosmid clone (pBHp53–2) carrying a segment from the same region of the genome on to combed P1 molecules by FISH with digoxigenin-labeled cosmid DNA and biotinylated P1 DNA. Figure 1D shows a typical hybridization result. The protocol deposited the high molecular weight P1 molecules at an average density of about one full length P1 molecule every one to two fields of view, so that image acquisition could be done rapidly. The region of homology spans about 39 ± 4 kb, based on length measurements of 11 individual molecules (Fig. 1E). Alignment of the images was facilitated by the presence of a region with high homology between cosmid and P1 DNA at one end of the fiber immediately flanking the *Not* site (Fig. 1D). A database search identified this region as a 493 bp stretch with 100% homology in both the p*Sac*II P1 and pWE15 cloning vectors. In addition to the region of homology (h) (Fig. 1D), we found several sites of weaker cross-hybridization between the cosmid and the P1 DNA caused by *Alu*-type repeats in the cosmid and P1 insert. The sites of cross-hybridization caused by *Alu*-DNA repeats showed up as small yellow spots along the fibers (Fig. 1D). The presence of *Alu*-type repeat elements in probe pBHp53–2 was further verified by hybridization of digoxigenin-labeled pBHp53–2 DNA to normal human metaphase spreads generating strong *Alu*-specific pattern (25) (data not shown). Furthermore, hybridization of a cloned *Alu*-DNA probe (PV92) (26) to fibers of clone RMC17P036 confirmed the localization of *Alu* repeat clusters on the P1 molecules (data not shown).

We explored the utility of quantitative DNA fiber mapping for longer range mapping by determining the precision with which two P1 molecules designated '1107' and '1143' (Fig. 2) could be mapped along the ~490 kb YAC clone '141G6' (26,27). These P1 molecules were known from STS content mapping to overlap with the YAC but not with each other (Fig. 2). Digoxigenin-labeled DNA from one or both P1 clones was hybridized to combed YAC molecules along with a probe for the YAC vector arms (pYAC3) that flanked each end of the YAC and detected using rhodamine-labeled antidigoxigenin. The YAC fiber itself was counterstained by hybridization with biotinylated total YAC DNA detected using avidin-FITC. This scheme allowed identification of full length YAC molecules as these carried red fluorescence signals at each end (produced by hybridization of the pYAC3 sequences) as well as localization of the P1 probes along the individual fibers.

The degree and uniformity of stretching achieved for the YAC molecules was assessed by measuring the lengths of the domains produced by hybridization with DNA from the ~81 kb P1 '1143' along 10 YAC fibers. The length of '1143' along the YAC fibers was 34.5 ± 2.55 μm, corresponding to stretching of 2.3 kb/μm, almost identical to that achieved for lambda phage. This suggests that the degree of stretching is highly reproducible and independent of the length of the combed molecule.

One important use of quantitative DNA fiber mapping is precise localization of probe DNA molecules along the target DNA molecule. A representative image of a hybridized full length (~490 kb) YAC in Figure 3A shows the locations of the two P1 probes, the extent of the gap between them and the terminal pYAC3 signals. Separate hybridizations of the two P1 probes showed that clone '1143' maps close to one end of '141G6', while clone '1107' maps close to the center of the YAC molecule (Fig. 3A,B). These results agree well with the map based on STS content mapping (Fig. 2).

The location of the P1 clone '1143' along the YAC was

Figure 4. Physical mapping of P1 clones on straightened YAC molecules using a triple color combination. DNA from clones '141G6', '1143' and '1107' were labeled with biotin, digoxigenin and FITC respectively, and detected in blue, red and green. The hybridization mixture also contained digoxigenin-labeled DNA from plasmid pYAC3 (red signal at the ends of the YAC molecules). The image shows a field of view of average DNA fiber density.

Figure 3. Physical mapping of P1 clones hybridized to YAC DNA molecules and determination of gap sizes. (**A**) Straightened molecules from YAC '141G6' (~490 kb, green) were hybridized with digoxigenin-labeled probes from P1 clones '1143' and '1107' (red) and the plasmid pYAC3 containing the vector arms (red). The figure shows a full length YAC molecule and numerous fragments. The hybridized P1 clones and pYAC3 binding sites as well as the gap between '1143' and '1107' are indicated. The resolution of the recorded image was reduced threefold to produce a figure of this size. (**B**) A typical YAC DNA molecule (fragment) shown at full resolution carrying the hybridization signals of plasmid pYAC3 and the two P1 probes '1143' and '1107'. The distances a and b are determined for physical mapping of '1143' on YAC '141G6'. The interval 'gap' represents the size of the gap region between the two P1 clones. (**C**) The average of 10 red fluorescence profiles along individual YAC molecules. Analysis of this average profile allowed determination of the gap size between P1 clones '1143' and '1107'. The hybridized P1 clones, the proximal pYAC3 binding site and the approximate locations of 50% values of the red fluorescence (dotted vertical lines) are indicated.

determined by measuring the distance of its hybridization signal from the end of the YAC marked by the pYAC3 signal (distance 'a' in Fig. 3B). The hybridization domain of clone '1143' began at 49.2 μm or 114 kb (±5.7 kb) from the proximal end of the YAC and extended 81 kb, assuming a conversion factor of 2.3 kb/μm. Measurement of the hybridization domain of clone '1107' suggested a mean size of 89.9 kb (±8.2 kb), which agrees well with the size of 88 kb obtained by pulsed field gel electrophoretic (PFGE) analysis. This conversion also allowed us to estimate the size of the YAC as 496 kb (standard deviation: 37 kb, $n = 4$). This is in good agreement with published values ranging from 430 to 495 kb (5,26,27).

Another important application of quantitative DNA fiber mapping is assessment of the extent of gaps in contig maps. The extent of the gap between clones '1143' and '1107', was determined to be 10.9 μm or 25.4 kb (±1.1 kb) by measuring the physical distances between the P1 hybridization signals on 10 YAC fibers. Partial fibers showing hybridization signals along the gap region and part of the flanking P1s were sufficient for determination of the size of the gap region as these all appeared to be equally stretched. Figure 3C shows the red fluorescence profile determined by averaging 10 individual profiles. The extent of the gap is readily apparent.

The construction of high resolution physical maps by quantitative DNA fiber mapping can be expedited by hybridization of several P1 clones labeled with different haptens and detected in different fluorescence wavelength intervals. We applied a triple color detection scheme by labeling the P1 clones '1143' and '1107' with digoxigenin and FITC, respectively, while the YAC '141G6' DNA was biotinylated. Bound probes were detected in red, green and blue, respectively. Figure 4 shows an image obtained using the triple color detection scheme and demonstrates the high density of DNA fibers per field of view. The density of fibers shown in Figure 4 was found optimal for analysis. We also found areas on the slides with an up to 10 times higher density of YAC DNA fibers, but those were more complicated to analyze due to fiber overlap. Fibers selected for analysis were straight molecules showing bead-like hybridization pattern as shown in Figure 3B for the dual color detection.

DISCUSSION

Quantitative DNA fiber mapping appears to be highly useful as a tool for construction of kilobase resolution physical maps comprised of minimally overlapping cloned DNA sequences. The sizes of the clones (DNA sequences) can be assessed by direct measurement of their lengths after molecular combing or by measuring the extent of their hybridization domains along stretched DNA fibers. The degree of overlap between elements of a contig can be assessed by quantitatively mapping overlapping clones, one on to another, or by mapping these clones along DNA fibers representing larger genomic regions. The highly reproducible stretching, which extends the DNA fibers to ~2.3 kb/μm, eliminates the need to scale each experiment using internal controls. On the other hand, hybridization experiments to determine the overlap between P1 clones, can include probes specific for the cloning vector sequences so that overlap can be immediately related to the orientation of insert (data not shown). Finally, clones can be mapped along YACs to determine order and to assess the extent of gaps between non-overlapping clones. This process is efficient as preparation of straight, uniformly stretched DNA fibers at high density is straightforward using molecular combing.

The high precision that can be achieved using quantitative DNA fiber mapping and the existence of extensive YAC contigs covering much of the human genome suggests a simple strategy for assembly of 'sequence ready' physical maps. The process begins with a selection of clones homologous to an element of a YAC contig (e.g. by hybridization of the YAC to an array of DNA fragments cloned into cosmids, P1s, BACs, PACs or other vectors suitable as substrates for sequencing) or by subcloning the YAC. Each selected clone is then mapped on to the YAC using quantitative DNA fiber mapping. The end result of this process should be a kilobase resolution map of the clones relative to each other, an estimate of the extent of overlap between them and an estimate of the sizes of gaps in the map. This information can then be used to guide gap filling (a critical component of map assembly). Short gaps might be crossed by PCR or by selection of clones from small insert libraries while longer gaps might be filled by selection of additional large insert clones. The development of techniques to microdissect the DNA fibers and preparation of probes by *in vitro* DNA amplification similar to the scheme applied for microdissection of metaphase chromosomes (29) is a further option for rapid gap closure as well as targeted generation of sequence tagged sites in the larger genomic interval defined by the high molecular weight DNA fiber.

Minor limitations in quantitative DNA fiber mapping at present arise from breakage of DNA during purification and molecular combing and the lack of automated fiber analysis software. The relatively low number of full length lambda molecules in our experiments may reflect the quality of the commercial preparation. High molecular weight DNA prepared from P1 and YAC clones by PFGE and agarase digestion in our laboratory, on the other hand, consistently yields a large number of undegraded DNA molecules, so that a single experiment, i.e. one coverslip, provides enough molecules for analysis. We estimate that the number of full length YAC or P1 molecules after molecular combing and FISH is in the range of a few percent, thus providing several hundred full length molecules per experiment, i.e. on average one intact molecule every two fields of view at 40×magnification. As shown above, only a few molecules are needed for quantitative DNA fiber mapping, and most mapping studies such as gap assessment or mapping relative to the proximal end of the YAC molecule can be performed rapidly and accurately on full length as well as fragmented DNA molecules.

The impact of quantitative DNA fiber mapping on genome research will depend on how well it can accommodate the needs of high throughput, large-scale genome sequencing projects. In preliminary experiments, we performed molecular combing on 20 individual DNA samples per slides arranged in four rows×five columns. The columns were separated by a thin border of rubber cement and DNA was dried under 5 mm wide strips cut from coverslips. First results showed that clone overlap can be detected at this scale (data not shown), and we are now optimizing the technique for routine mapping. Development of semi-automated slide scanning and image acquisition should facilitate these aspects of the analysis procedure. These developments should bring quantitative DNA

fiber mapping to the point where it is of major utility in assembly of sequence ready physical maps.

MATERIALS AND METHODS

Slide preparation

Slides were cleaned in equal parts of concentrated hydrochloric acid and methanol for at least 30 min followed by overnight immersion in 18 M sulfuric acid. The slides were then washed in eight to 10 changes of ultrapure water, immersed in boiling water for 10 min and air dried. Cleaned slides were placed in a 10% solution of 3-aminopropyltriethoxy silane (APS, Sigma, St Louis, MO) in 95% ethanol for 1 h, rinsed several times with water, washed with ethanol and air dried. Prior to use, slides were heated to 80–110°C overnight.

DNA preparation and molecular combing

Approximately 10^7 lambda DNA molecules (Sigma, St Louis, MO) were stained with 0.5 µM YOYO (30) and then resuspended in 1% *p*-phenylenediamine, 15 mM NaCl, 1 mM H_2PO_4, pH 8.0, 90% glycerol (AF-solution) (24). This suspension reduced DNA breakage and allowed the samples to be stored at room temperature for 24 h with minimal breakage. However, it also substantially reduced binding to the solid substrate and the high glycerol concentration prevented drying. Further dilution of this suspension with water at a ratio of 1:10, allowed DNA binding and permitted good combing when the slide was dried at room temperature.

One microliter of lambda DNA containing approximately 10^6 molecules in diluted AF-solution was placed on an APS-derivatized microscope slide, covered with a round 18 mm coverslip and allowed to dry overnight at 20°C.

We isolated a large insert DNA clone from a human P1 library by PCR screening (31). Clone RMC17P036 has a total size of approximately 95 kb and contains an insert of approximately 78 kb including exon 5 and exon 8 of the human p53 gene. DNA from clone RMC17P036 was isolated by an alkaline lysis procedure, linearized with *Not*I and purified by PFGE. Bands were excised and gel slices were treated with agarase (2 units in 200 µl agarase buffer, New England Biolabs, Beverly, MA). An aliquot of 1 µl containing approximately 0.5–1 ng of DNA was mixed with 1 µl of 10^{-6} M YOYO and 9 µl of AF-solution and diluted 1:10 with water. Molecular combing was performed as described above.

The P1 clones '1107' and '1143' were isolated from a human genomic P1 library (4) in the course of assembly of a P1-based map by STS content mapping (J-F.Cheng, unpublished). The sizes of the human genomic insert in P1 clones were calculated from the PFGE analysis of *Not*I linearized recombinant P1 molecules. The size of the recombinant cloning vector p*Sac*II P1 is ~17 kb.

High molecular weight YAC DNA from clone '141G6' was isolated by field inversion gel electrophoresis using a BioRad CHEF Mapper II PFGE apparatus according to the manufacturer's instructions. Southern blot analysis of clone '141G6' performed in our laboratory indicated a YAC size of ~475 kb. Slices containing the YAC were excised from the low melting point agarose and equilibrated in agarase buffer. Agarose was digested with agarase and YAC DNA was dialyzed for 2 h against 100 mM NaCl as described (32), except that a Millipore Minitan-S polysulfone membrane filter (100 000 NMWL) was used for microdialysis. The YAC DNA was diluted with water to 1.2 ng/µl and mixed with an equal amount of YOYO (10^{-6} M). After a 3 min incubation at 20°C, the DNA was diluted a further 1:10 with water. Two microliters of this DNA containing approximately 24 pg or 30 000 full length molecules was placed on a non-treated 22×22 mm coverslip. A silanated slide was then placed on top and the liquid was allowed to dry at 4°C overnight.

DNA probes

Products from digestion of lambda DNA with *Hin*dIII were purified by gel electrophoresis and labeled by random primer extension incorporating either biotin- or digoxigenin-dUTP. The 9.4 and 23 kb *Hin*dIII fragments were biotinylated, while the 4.3 and 6.6 kb fragments were labeled with digoxigenin.

Labeled P1 probes were generated from DNA prepared from overnight cultures of clones RMC17P036, '1143' and '1107' by an alkaline lysis protocol (31). Aliquots of 400 ng of DNA from the P1 clones were labeled by random priming incorporating biotin-dUTP (RMC17P036) or digoxigenin-dUTP ('1143' and '1107'). Cosmid DNA (clone pBHp53–2, kindly provided by Dr R.White) was isolated by the same procedure and random primed in the presence of digoxigenin-dUTP.

The biotinylated DNA probe for counterstaining the YAC molecule was made by random priming of DNA isolated from the YAC containing yeast clone '141G6'. Digoxigenin-labeled DNA from pYAC3 (a plasmid containing both YAC vector arms) was included in the hybridization probe mixtures specifically to label the ends of untruncated YAC molecules. Our dual color detection scheme reveals regions of overlap as red/yellow stained regions, while parts of the YAC such as flanking sequences that do not bind the pYAC3 plasmid or the P1 probes appear in green. It should be noted that *Cot*-1 DNA, normally employed to block hybridization of repeated DNA sequences during FISH with large-insert human probes (31), is not needed in this protocol because the repeats are widely distributed along the combed DNA fibers.

FISH

Approximately 20 ng of each probe in 10 µl of 55% formamide, 10% dextran sulfate, 100 ng/µl salmon sperm DNA, in 300 mM NaCl, 30 mM Na citrate, pH 7.0 (2×SSC), was applied to the slide and coverslipped. Denaturation was done at 100°C for 3 min, and the probe was allowed to hybridize overnight at 37°C. The slides were then washed in three changes of 2×SSC at 20°C for 15 min each with slight agitation. Bound probes were detected by conjugation with avidin-FITC (Vector, Burlingame, CA) and a rhodamine-labeled sheep antibody against digoxigenin (Boehringer Mannheim (BM), Indianapolis, IN) (33). Slides were washed twice in 2×SSC and hybridization signals were amplified with biotinylated goat-anti-avidin (Vector) followed by a second layer of avidin-FITC and a Texas Red-labeled rabbit antibody against sheep IgG (Vector) (34,35). Slides were finally washed twice in 2×SSC and mounted in AF-solution for microscopic inspection.

In the triple color detection scheme, we hybridized biotinylated YAC DNA in combination with digoxigenin-labeled probes for pYAC3 and the P1 clone '1143'. The digoxigenin-labeled probes were detected in red as described above. The biotinylated YAC probe was visualized by incubation with AMCA avidin D (Vector) (blue) followed by two signal amplification steps using the biotinylated goat-anti-avidin antibody. The probe for P1 clone '1107' was labeled with FITC by random priming incorporating FITC-dUTP (BM), and detection was performed with an anti-FITC antibody (raised in mouse, DAKO, Carpintera, CA) followed by incubation with an FITC-conjugated horse–antimouse antibody (Vector).

Image analysis

Images were acquired on a quantitative image processing system based on a Zeiss fluorescence microscope equipped with 63×, 1.25 NA and 40×, 1.3 NA oil objectives, a Photometrics cooled CCD camera, multibandpass filters for simultaneous observation of FITC and Texas Red or AMCA/DAPI (Chroma Technology, Brattleboro, VT) and a SUN SPARC workstation (36). Images of stretched lambda molecules of about 21.6 µm were acquired at 63× magnification. Forty-five molecules of approximately full length and without overlap with other DNA fibers were selected for analysis. The longitudinal fiber axis was traced interactively and a computer program calculated integrated fluorescence for 9 pixel wide cross-sections equivalent to 1.0 µm along the selected path. Fluorescence profiles were aligned manually based on the minimum of red fluorescence in the 9.4 kb segment (Fig. 1C). Profiles were averaged and the position of the 10, 50 and 90% values were calculated for the peaks in the longitudinal fluorescence profiles. Map positions were determined by first calculating the ends of the linear molecules. We defined points where the fluorescence of the flanking green fragments had decreased to 10% of its peak value as molecule endpoints giving a measure of 200 pixels for the lambda molecule length and a conversion factor of 2.26 kb/µm. Pixel spacing was 0.1079 µm/pixel. We then used the positions where the fluorescence of the 4.3, 9.4 and 6.6kb fragments had reached 50% of its value to define the boundaries of the labeled fragments. The difference between the measured boundaries and exact position of *Hin*dIII restriction sites was defined as the accuracy of the measurement. The interval between the 10 and 90% values was defined as mapping precision. The size of the labeled 4.3, 6.6 and 9.4 kb fragments was calculated from the physical distance between fragment boundaries (the 50% values) multiplied by 2.26 kb/µm.

Images from P1 and YAC molecules were recorded using the 63× and 40× lens, respectively, and analyzed as described above for lambda DNA molecules. The pixel spacing for the 40x lens is 0.17 µm/pixel. The image shown in Figure 4 was contrast enhanced using the high pass filter function (width: 10 pixels) of Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA).

Sequence analysis

Alignment of the sequences of the pSacBII P1 cloning vector (GenBank accession number U09128) and pWE15 cosmid vector DNA (accession number X65279) using MacVector (Kodak, New Haven, CT) indicated a region of high homology approximately between base position 15 000 and 16 000 of the p*Sac*BII P1 vector. A homology search using the daily update of

the Genome Sequence Database (GSDB-LANL) identified a 493 bp region of pWE15 with 100% (491/493) homology ending 46 bp upstream of the *Not*I site in p*Sac*BII P1. The human p53 gene (GenBank accession number X54156) contains several clusters of *Alu*-type repeats.

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